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USDA-ARS Plant Science Research Unit, Department of Agronomy and Plant Genetics, University of Minnesota, St Paul, MN, USA

Germplasm Variability and Environmental Effects on Stem Cellulose and Lignin Concentrations in Alfalfa*

P. M. Schwab, J. F. S. Lamb, C. C. Sheaffer, and D. K. Barnes

Authors' addresses: Dr P. M. Schwab, Cooperative States Research, Education and Extension Service, 1400 Independence Ave, Washington, DC, USA; Dr J. F. S. Lamb (corresponding author; e-mail: lambx002@umn.edu), USDA-ARS Plant Science Research Unit, Department of Agronomy and Plant Genetics, University of Minnesota, St Paul, MN 55108, USA; Dr C. C. Sheaffer, Department of Agronomy and Plant Genetics, 411 Borlaug Hall, University of Minnesota, St Paul, MN 55108, USA; Dr D. K. Barnes, USDA-ARS Plant Science Research Unit, Department of Agronomy and Plant Genetics, 411 Borlaug Hall, University of Minnesota, St Paul, MN 55108, USA

With 5 tables

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Abstract

Stem cellulose and lignin concentrations are major determining factors of alfalfa (*Medicago sativa* ssp. *sativa* L.) forage quality. Only limited information is available on the genetic variability and the influence of environmental effects on these two stem-quality traits. Our objectives were to: evaluate the variability for stem cellulose and lignin concentrations in modern alfalfa germplasms across several harvests; observe the environmental stability of these two quality traits in 32 alfalfa clones selected high or low for either stem cellulose or lignin concentration; and examine the relationships between these two stem-quality traits and leaf and stem crude protein (CP). Fifty alfalfa entries (cultivars and experimental populations) were established May 1993, and sampled for stem acid-detergent lignin (ADL), stem acid-detergent cellulose (ADC), and stem CP on Sep 1993, June and Aug 1994. Clones were vegetatively propagated from individual plants selected for extremes in stem ADL and ADC and transplanted into blocks at two locations in May 1994 and sampled for quality analysis in September 1994. For all samples, leaves were hand-separated from stems and stem ADL, ADC, CP and leaf CP concentration were determined using near infrared reflectance spectroscopy. Entry differences for stem ADL and ADC were detected only at the June 1994 harvest date in the cultivar study. Spearman's ranked correlations over years in the clonal study demonstrated greater environmental stability for stem ADC ($r = 0.70$, $P \geq 0.01$) than for stem ADL ($r = 0.54$, $P \# 0.05$). Environmental effects had an impact on both traits, but stem ADC showed greater potential for improving forage quality. Simple correlations showed that decreasing stem ADL or ADC would have minimal effect on leaf CP and may increase stem CP.

Key words: alfalfa — forage quality — lucerne — *Medicago sativa* ssp. *sativa* L

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Introduction

The forage quality of stems affects the overall quality of alfalfa herbage. Alfalfa stems constitute ≥50 % of the total alfalfa herbage and contain greater concentrations of lignin and cellulose and less crude protein (CP) than leaves. The secondary cell wall of alfalfa stems is composed of up to 150 g kg^{-1} lignin and 700 g kg^{-1} cellulose on a dry weight basis (Marten et al. 1988). In addition, the quality of stems decreases more rapidly with advancing maturity than that of leaves (Kilcher and Heinrichs 1974, Albrecht et al. 1987). Increases in lignin content have been reported to cause substantial reduction in the digestibility of forage (Buxton and Casler 1993). Selection for reduced stem lignin has been offered as a possible method for improving forage quality in alfalfa used for animal feed (Buxton et al. 1987).

Most forage-quality research conducted in alfalfa has been on the whole herbage, and not the stem and leaf portions. Coors et al. (1986) selected

plants with high whole-herbage CP and low acid-detergent fibre (ADF) from experimental populations. The selected populations differed from the parental populations for whole-herbage ADF, acid-detergent lignin (ADL) and CP, but not for ADC. They concluded that selection for low ADF reduced both total cell wall and ADL concentration. Shenk and Elliot (1970) reported that selection for increased digestibility of the whole herbage decreased cell wall constituents and ADL. Kephart et al. (1989) reported success in selecting for divergent ADL concentration in whole herbage of experimental populations and concluded that many of the differences observed in the divergent ADL lines may have been due to correlated changes in leaf to stem ratio. Differences for percent whole-herbage lignin were apparent between selected lines, but these differences were not consistent across all harvests or locations. Leaf to stem ratio was greater in the low ADL lines than in the high ADL lines. Subsequent studies analysing only stem tissue showed no difference in stem ADL between these populations selected for high and low whole-herbage ADL (Kephart et al. 1990). Hill and Barnes (1977) examined the genetic variability for several forage-quality traits for whole herbage in several alfalfa germplasms and found positive correlations between ADF and ADL and negative correlations between CP and either ADF or ADL. Both Shenk and Elliot (1970) and Hill and Barnes (1977) concluded that as no quality parameters were closely correlated with yield, improvement for both yield and quality in alfalfa should be possible.

Seasonal and year-to-year environmental variation can affect the forage quality of alfalfa (Marten et al. 1988, Buxton and Casler 1993, Sheaffer et al. 1998). Forage from the first or spring harvest of alfalfa is generally of lower forage quality than later harvests during the growing season because spring-harvested forage has a lower leaf to stem ratio than summer harvests. Spring-harvested forage also decreases in quality with increasing maturity to a greater extent than subsequent harvests. Drought stress increases leafiness and digestibility by decreasing cell wall and lignin concentrations. Warm weather generally decreases digestibility without consistently affecting the crude fibre, lignin, or cellulose concentrations. Buxton and Casler (1993) reported that cell wall lignin was more environmentally stable than other forage-quality traits. The extent of environment influence on the forage quality of alfalfa genotypes is not known, although few cultivar × environment interactions have been reported.

Some researchers have focused on the forage quality of just the stem portion of alfalfa herbage. Buxton et al. (1987) compared forage quality in the stems of 64 plant introductions and five cultivars of alfalfa and found variation among alfalfa introductions and cultivars for stem cell wall lignin, stem *in vitro* digestable dry matter (IVDDM), and stem CP. Harvest and harvest × entry interaction effects were not significant for stem lignin concentration in their experiments, suggesting that stem cell wall lignin was stable over harvests. Stem cell wall lignin was not affected by the stage of maturity, whereas stem IVDDM and stem CP were. Stem IVDDM and stem CP were positively correlated with one another but negatively correlated with stem cell wall lignin concentration. Alfalfa stems harvested in the spring have lower CP, cell wall constituents, and lignin, and higher neutral detergent fibre (NDF) than those harvested in the summer (Sanderson and Wedin 1988, Sheaffer et al. 2000). At later stages of maturity, alfalfa stems increase in ADF, NDF, and ADL and decline in CP and cellulose concentration (Sanderson and Wedin 1988, Sheaffer et al. 2000).

It has generally been assumed that there is little relationship between the quality of leaves and stems although little data exist to support this assumption (Marten et al. 1988). Additionally, only sparse knowledge exists concerning the stability of leaf- and stem-quality relationships across harvests and environments. Information on environmental stability of stem quality is needed for plant breeders wishing to select cultivars with improved forage quality.

Our objectives were to: (1) observe the variability of stem and leaf forage-quality components among modern alfalfa germplasms across several harvest dates; (2) examine the relationships between stem and leaf quality components and (3) examine the environmental effects on stem ADL, ADC and CP of 32 individual vegetatively propagated alfalfa clones which were selected for extremes in stem ADL and ADC based upon a single observation.

Materials and Methods

Cultivar/germplasm experiment

Twenty-eight commercial cultivars and 22 experimental lines chosen to represent the range of germplasm grown in the upper midwest United States (Table 1) were planted at the University of Minnesota Research and Outreach Center, Rosemount, MN [Tallula silt loam, (coarse-silty, mixed,

Table 1: Mean values of stem acid-detergent lignin (SADL), stem acid-detergent cellulose (SADC), stem crude protein (SCP), and leaf CP (LCP) for 50 alfalfa cultivars and experimental entries averaged over three harvest dates (September 1993, June 1994 and August 1994) at Rosemount, MN

Entries	SADL	SADC	SCP	LCP
Commercial cultivars				
636	111	423	116	289
5262	116	425	112	286
5364	114	427	110	285
5454	114	422	115	281
5472	116	432	106	271
Agate	116	429	109	283
Alfa	119	437	103	279
Alfagraze	115	430	114	289
Apollo Supreme	117	434	105	284
Arc	117	426	112	287
Cimarron	114	430	117	289
DK 120	114	422	113	290
Epic	114	427	114	283
Flagship75	115	433	109	288
Good as Gold	115	428	113	289
Jade	116	432	109	286
Legend	113	418	117	285
Magnum III	117	424	114	293
Mohawk	111	418	120	291
MultiKing1	110	428	113	290
Multiplier	118	437	105	287
Orca	117	433	101	277
Profit	114	423	113	284
Saranac-AR	112	428	111	286
Vernal	112	430	111	277
Vernema	114	418	111	280
Webfoot	110	416	117	283
WL 322HQ	113	414	118	296
Experimental entries				
2B07	114	418	114	281
2J01	116	424	117	292
2J08	113	427	118	284
2J11	113	419	117	282
3B04	112	425	115	287
3B27	117	425	113	278
3B36	115	430	115	286
3B51	113	426	116	295
4J10	115	429	111	285
4J19	115	426	111	281
90SY5V2	114	423	116	280
90W3PR1	114	435	108	285
91IO1PV1	115	425	112	283
92N02CL2	116	425	117	285
92W02PM1	111	421	117	285
MNGRN-14	110	429	114	284
MWNC4	113	433	108	279
P90CR11	114	427	114	287
P90CR12	116	429	108	287
W92CM84	117	431	114	286
XAE11	116	432	110	284
YAM91	114	424	114	281
LSD 0.05	5	12	8	8
CV %	5	3	9	3

Data are least square means of four replicates and three harvest dates (g kg^{-1} dry weight).

mesic Typic Hapludoll)] on 15 May 1993. All 50 entries were seeded at a rate of 50 live seeds m^{-1} into single-row plots 3 m long with 0.6 m between plots and 1 m spacing between rows. The experimental design was a randomized complete block with four replications. Plots were clipped on 20 July 1993 when the plants had reached early flowering stage (stage 5; Kalu and Fick 1981) and the herbage was discarded. Plots were harvested at late flower to early pod development (stage 6; Kalu and Fick 1981) on 10 September 1993, 24 June 1994 and 18 August 1994. Twelve stems which had reached stage 6 were randomly chosen for forage-quality analysis from the interior of each plot.

Clonal plant experiment

The experimental cultivar MWNC4 (USDA-ARS, University of Minnesota), a 163-clone synthetic selected for nematode and multiple disease resistances, was used in this experiment. Experimental plants were seeded in greenhouse sandbenches on 9 April 1993. Two thousand plants were transplanted to the field at the University of Minnesota Research and Outreach Center, Rosemount, MN on 15 May 1993. Plants were space-planted into blocks containing 100 plants each in a 10×10 grid with 15 cm between plants within blocks and 1 m alleys between blocks. The plants were clipped on 20 July and the herbage discarded. Plants were allowed to regrow until most plants had reached late flowering (stage 5; Kalu and Fick 1981). Only plants having reached stage 5 by 5 September were harvested for forage-quality analysis. Stems were analysed from all 2000 plants and 32 individual plants were identified to represent the extremes in stem ADL and ADC and brought into the greenhouse to overwinter. Eight plants were chosen for having high lignin concentration, eight for low lignin concentration, eight for high cellulose concentration, and eight for low cellulose concentration. Ramets of these selected plants were vegetatively propagated in spring 1994. Stem cuttings were rooted in vermiculite and the clones were transplanted to containers in the greenhouse. The experimental germplasm 'MNGRN-14' (University of Minnesota, USDA-ARS) was seeded into containers at the same time.

The clones were transplanted into four replicate blocks on 9 June 1994 at the University of Minnesota Sand Plains Experiment Station, Becker, MN [Hubbard loamy sand (sandy, mixed Udorothentic Haploborall)] and 10 June 1994 at the University of Minnesota Research and Outreach Center, Rosemount, MN. Each block was established in a

4×8 grid with 15 cm between plants. Clones of each genotype were randomly assigned to a position within each block. Plants of MNGRN were transplanted to surround each block as a border with 1 m alleyways between blocks. All blocks were clipped in mid-July 1994 and the herbage was discarded. Plants were harvested on 24 August and 1 September at Becker and Rosemount, MN, respectively, and stems were analysed for ADL and ADC.

Forage-quality analysis

All forage-quality samples were oven-dried at 60 °C. Leaves and stems were hand-separated and stems were coarse-ground through a 4-mm screen in a Wiley mill before being ground through a 1-mm screen in a cyclone grinder. Leaves were ground through a 1-mm screen in the cyclone mill. Reflectance data from ground leaf and stem samples were collected using near-infrared reflectance spectrometry (NIRS) on a NIRS Systems model 6500[1] (Infrasoft International, Silver Springs, MD) scanning monochrometer. Stem ADL, ADC, CP and leaf CP were predicted from NIRS equations (Marten et al. 1989). Only stem-quality components were measured in 1993 in the cultivar study. Laboratory chemical analysis was performed on a subset of the samples for calibration of the NIRS equations. Laboratory analysis of ADL and ADC content followed the procedures of Goering and Van Soest (1970). Crude protein calibration analysis was determined by the micro-Kjeldahl method. The reflectance data were related to the calibration data using a modified partial least squares regression procedure (Shenk and Westerhaus 1991, Infrasoft International Inc 1992). Equation statistics are presented in Table 1 for both experiments.

Statistical analysis

Analysis of variance (Proc GLM; SAS Institute 1991) was conducted to determine significant variation due to harvest and entry. Experiments were analysed as randomized complete block designs. Entries were considered fixed and harvests and replicates were considered to be random factors. The harvest mean square was tested for significance using the harvest × replicate interaction term. Mean separation was performed using Fisher's LSD procedure only if significant treatment mean squares were detected. Pearson's and Spearman's correlation coefficients were calculated using Proc CORR (SAS Institute 1991) to determine relationships between traits.

Results and Discussion

Cultivar/germplasm experiment

Entries (cultivars and experimental germplasms) differed for stem ADL, stem ADC, stem CP and leaf CP when values were averaged over the three harvest dates (Table 1) In September 1993, no differences among entries were detected for stem quality, but entries differed for leaf CP. Entries varied for all quality traits at the June 1994 harvest while no variability among entries was found for any of the quality traits at the August 1994 harvest (data not shown). Buxton et al. (1987) observed differences among entries at some harvest dates but not at others when examining forage quality among alfalfa plant introductions. Experimental entries as a group were similar to commercial cultivars for all stem- and leaf-quality characteristics. Some changes in entry rank were observed for stem ADL at the different harvest dates. Stem ADL in 'Orca' varied from 111 g kg^{-1} dry weight (49th of 50 entries) at the September 1993 harvest to 125 g kg^{-1} dry weight (first) at the June 1994 harvest to 115 g kg^{-1} dry weight (second) at the August 1991 harvest.

The ranges and standard deviations of most forage-quality traits were largest at the June 1994 harvest date, and in general were smallest at the August 1994 harvest date (Table 2). Marten et al. (1988) also reported wider ranges in forage quality at the first harvest compared with later harvests in the growing season. Sheaffer et al. (1998) and Marten et al. (1988) also reported a greater range in entry means at spring harvests compared with summer harvests. Time of harvest during the growing season appears to have considerable influence on stem quality traits in alfalfa. Differences among entries only at the June 1994 harvest date for all three stem-quality traits suggests that selection for these traits would be more feasible when conducted at harvests early in the growing season.

Mean stem ADC (averaged over all entries) was lower in August 1994, compared with the other two harvest dates which were similar (Table 2). Mean stem CP was greatest at the August 1993 harvest, followed by June 1994, with September 1993 the lowest. Mean leaf CP was similar in September 1993 and August 1994 with June 1994 being lower. Sheaffer et al. (1998) stated that whole-herbage CP was lowest at the spring harvest. Our results agree for leaf CP but not for stem CP. In contrast to our observations, Buxton and Casler (1993) reported that forage-quality components in forage species were stable across harvest environments. Some of the disparities between the results from 1993 and 1994 harvests may be attributable to the unusually cool and wet summer and autumn of 1993 or the intrinsic differences in forage quality between

[1]Mention of a specific instrument does not imply its endorsement by the University of Minnesota or USDA-ARS.

Table 2: Ranges, means and standard deviations (S.D.) of stem acid-detergent lignin (SADL) stem acid-detergent cellulose (SADC), stem crude protein (SCP), and leaf crude protein (LCP) of 50 alfalfa entries harvested on three dates at Rosemount, MN

	SADL			SADC			SCP			LCP		
Harvest date	Range	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range	Mean	SD
September 1993	110–127	117	3.2	428–463	445	7.7	84–106	97	4.7	279–309	296	6.9
June 1994	108–125	115	3.4	412–449	431	8.3	90–126	112	6.9	253–279	267	5.4
August 1994	104–116	111	2.6	385–420	404	7.1	116–139	128	5	280–311	292	5.8
LSD (0.05)		5			12			4.4			10	

Data are g kg^{-1} dry weight.

establishment and first-production year alfalfa stands.

Stem ADL and ADC, were positively correlated with one another indicating that selection for changes in one of these traits would result in changes in the other (Table 3). Both of these traits were negatively correlated with stem CP at all harvest dates suggesting that any selected decreases in stem ADC or ADL would result in increasing stem CP concentration. Hill and Barnes (1977) reported similar phenotypic correlations between total-herbage CP and total-herbage lignin. No correlation was evident between leaf CP and either stem ADC or ADL which indicated that selection for decreasing stem ADC or ADL should have very little effect on leaf CP concentration. A small positive correlation was found between leaf and stem CP suggesting that any increases in one of these two traits should increase the other.

Table 3: Pearson correlation coefficients describing the relationships between stem acid-detergent lignin (SADL), stem acid-detergent cellulose (SADC), stem crude protein (SCP), and leaf CP (LCP), in 50 alfalfa cultivars and experimental entries harvests at three dates in 1993 and 1994

Trait	SADC	SCP	LCP
September 1993			
SADL	0.69**	−0.50**	NS
SADC		−0.44**	NS
SCP			0.36**
June 1994			
SADL	0.47**	−0.58**	NS
SADC		−0.82**	NS
SCP			0.14*
August 1994			
SADL	0.45**	−0.56**	NS
SADC		−0.85**	NS
SCP			0.19**

*, **Represent 0.05 and 0.01 levels of significance, respectively.

Clonal plant experiment

In 1993, the group of plants selected for high stem ADL or for high stem ADC were separable from the group of plants selected for low stem ADL or low stem ADC, respectively (Table 4). Vegetatively propagated clones of the high and low stem ADL selections evaluated in 1994 did not segregate into the high and low groups as selected in 1993. Some clones identified as high in stem ADL in 1993 were low in stem ADL and vice versa in evaluations conducted in 1994. Consequently, the Spearman's rank correlation between 1993 and 1994 for clones selected for stem ADL was moderate ($r = 0.54$, $P \# 0.05$). We were not able to successfully identify groups of alfalfa plants that were consistently high or low in stem ADL when evaluated in different environments. Kephart et al. (1989) reported successful segregation between high and low lignin populations in only one of two germplasms sources in which selection was conducted.

Differences between high and low stem ADC selected groups evaluated in 1994 were not as great as those identified in 1993, but the high and low groups were still distinguishable. Clones identified as either high or low in stem ADC in 1993 remained in the same selected groups at both locations in 1994 and the Spearman's rank correlation between the 2 years was moderately high ($r = 0.70$, $P \# 0.01$). These results indicated repeatability of performance over environments and demonstrates potential for genetic improvement for stem ADC.

Location differences were observed for stem forage quality traits in 1994, but no clone × location interactions were found (data not shown). However, clones grown at one location had higher

Table 4: Ranges, means and standard errors of eight clones selected either high or low for either stem ADL or stem ADC the fall of 1993, and replicated means of vegetatively propagated clones of the selections over two locations in the fall of 1994

Clonal selection	September 1993						September 1994					
	SADL			SADC			SADL			SADC		
	Range	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range	Mean	SD
High SADL	121–142	130	3				114–129	119	3			
Low SADL	91–104	100	2				102–123	112	4			
High SADC				471–514	489	5				441–472	452	4
Low SADC				401–417	411	2				411–437	423	3

Data are g kg^{-1} dry weight.

stem ADC concentration and lower stem CP than plants grown at the second location. The overall range in stem ADL and stem ADC concentrations across all clones in both locations in 1994 was less than half that identified in 1993 (data not shown).

All clones selected for either stem ADL or stem ADC were evaluated for stem CP in both 1993 and 1994. Stem CP over both locations in 1994 (mean = 110 g kg^{-1}) was greater than in 1993 (mean = 94 g kg^{-1}), but the range in stem CP was similar between the 2 years. Spearman's rank correlations between 1993 and 1994 for stem CP for all 32 clones was moderate ($r = 0.56$, $P \# 0.01$). These results indicated some shifting in rank among the clones for stem CP between the 2 years, suggesting some influence of environmental effects for the expression of stem CP in this set of alfalfa clones.

Association among the leaf and stem forage-quality traits between the two locations in the clonal study were similar to those found in the cultivar study. Stem ADL and stem ADC, were positively correlated with one another and both of these traits were negatively correlated with stem CP (Table 5). These correlations suggest that any selected changes in either stem ADL or stem ADC would affect the other, and decreases in either of these traits would increase stem CP concentration. Once again, these results agree with those of Hill and Barnes (1977) who reported negative correlations between total-herbage CP and total-herbage lignin. Small negative correlations between leaf CP and either stem ADC or ADL indicated that selection for decreasing stem ADC or ADL could increase leaf CP concentration. Again, as in the cultivar study, leaf and stem CP were weakly positively correlated which suggested a limited relationship between leaf and stem CP, but any increases in one of these two traits could increase the other.

Table 5: Pearson correlation coefficients describing the relationships between stem acid-detergent lignin (SADL), stem acid-detergent cellulose (SADC), stem crude protein (SCP), and leaf CP (LCP) in 32 vegetatively propagated alfalfa clones harvested at two locations in September 1994

Trait	SADC	SCP	LCP
SADL	0.59**	−0.61**	−0.22**
SADC		−0.80**	−0.20**
SCP			0.36**

**0.01 level of significance.

Conclusions

Entry differences for stem ADL, stem ADC and stem CP were demonstrated in the cultivar study. Separation among the entries for these stem quality traits at only one harvest date in cultivar study, indicated that environmental effects have a large impact on stem ADC, stem ADL and stem CP in alfalfa. Ranked correlations over years in the clonal study demonstrated repeatability and perhaps greater environmental stability for stem ADC than for stem ADL. Clones selected for high or low stem ADC did remain in the originally selected high or low groups when evaluated in different environments. Variability among cultivars, and moderately high rank correlation among clones from one year to the next for stem ADC demonstrates potential for focusing on this trait in a plant breeding program to improve alfalfa forage quality. Positive correlations between stem ADC and stem ADL suggested that selection for changes in stem ADC could produce similar changes in stem ADL. Other simple correlations implied that decreasing stem

ADC or stem ADL would have minimal effect on leaf CP and could increase stem CP.

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